Chapter 8

General Discussion
Nasopharyngeal carcinoma (NPC) is a malignancy with distinct ethnic and geographical distribution.¹ This tumor is relatively rare in Caucasian, Hispanic and North-Asian populations but presents a huge burden in Southern China and Southeast Asia. The annual incidence rate is less than 1/100,000 population in non-endemic areas, 5-17/100,000 in Southeast Asian countries and about 20-50/100,000 in highly endemic Southern China areas²-⁶ Since there is no appropriate cancer registry in Indonesia, NPC incidence rate was derived from pathology-based data in local province of Yogyakarta in 1998 showing rate of 6.2/100,000 population per year,⁷ which is confirmed in the registry of 13 university hospitals in Indonesia. (Kurniawan and Cornain, pers. communication; Adham et al., submitted) This makes NPC the most prevalent head and neck cancer and the fourth form of cancer overall in man. When extrapolated to the whole 230 million person population of Indonesia, approximately new 12,000 cases can be expected yearly, making this a medical problem for the country. However, most NPC cases are not recorded, due to limitations in NPC awareness, poor facilities for diagnosis and lack of a nationwide cancer registry.

The majority of newly diagnosed NPC cases present with loco-regionally advanced disease, most commonly with cervical nodal metastasis. Currently, with the international standard of care consisting of concurrent chemo-radiotherapy with cisplatin-based regimens, 5-year overall survival can be achieved in 78% cases with conventional radiotherapy and 85% with intensity-modulated radiation therapy (IMRT).⁸ At early stages of NPC radiotherapy alone can achieve a 5-year overall survival of 90%, especially when IMRT is used.⁹ Unfortunately, in the last 5 years 86% patients in our local hospital came at late stage with progressive disease (Hariwiyanto et al., 2011, unpublished data) associated with high a rate of treatment complications, recurrence and mortality. (Kurnianda et al., unpublished data) (Wildeman et al., in preparation)

The poor outcome of NPC in Indonesia has many causes, such as lack of primary prevention (identification and elimination of risk factors), lack of secondary prevention (awareness and screening risk-groups for early-stage tumor identification), high rate of tumor progressivity and lack of effective treatment (especially lack of radiotherapy facilities) and high rate of progressivity and recurrence of the disease itself. Currently in Indonesia, secondary prevention is the most relevant preventive medical approach because of the clinical and biological feasibility to identify NPC cases at earlier stage. The goal of NPC screening is to detect cancer early enough so that treatment can be curative or significantly prolong survival. Increasing awareness on signs and symptoms of early stage NPC and implementation of novel diagnostic options with selection of suitable groups at risk for NPC is considered essential for a screening program.

Although the etiology of NPC is complex, it is widely accepted that Epstein-Barr virus (EBV) infection is a crucial factor in NPC pathogenesis either in endemic or nonendemic areas.¹⁰ The association between NPC and EBV was established by serological studies and later confirmed by demonstration of EBV DNA and
nuclear antigen proteins (EBNA) in NPC tumor cells.\textsuperscript{11,12} The notion that EBV plays an important role in the development of NPC is further supported by the observation that premalignant nasopharyngeal lesions (dysplasia and carcinoma \textit{in situ}) are already EBV-positive, carrying latent and clonal viral genomes as well as viral oncoproteins such as LMP1.\textsuperscript{13} EBV is associated with many malignancies including Burkitt’s lymphoma, Hodgkin disease, extra nodal T/NK cell lymphoma, immunoblastic B cell lymphoma in immunocompromized individuals, gastric carcinoma and NPC, classifying the virus as a human class I carcinogenic agent.\textsuperscript{14} The different viral activities (latency, default and growth program) seen in these various EBV-associated malignants reflect the dual cell tropism of EBV and distinct stages of EBV persistence in the human host.\textsuperscript{15}

With rare exceptions, most adults throughout the world harbor EBV for life. Although this virus was shown to induce immortalization and proliferation of B lymphocytes, lymphoid malignancies are rare and the virus carrier state is generally symptomless. The harmonious co-existence between host and EBV is the result of a long co-evolution with mutual adaptation, based on variation in the viral gene expression in different cell types and the finely tuned immune response of the host.\textsuperscript{16} When the immune system is impaired, such as by the use of immunosuppressive agents or in case of HIV infection, EBV positive cells may grow out resulting in reversible lymphoproliferative disorders which may develop to aggressive malignant lymphoma when left untreated.\textsuperscript{17} In the immune-competent host, non-viral molecular abnormalities imposed by the host or environment such as genetic and epigenetic changes, exposure to chemicals, including agents and conditions that can activate EBV, increases the possibilities for EBV-driven tumors to grow.\textsuperscript{18,19} Most interestingly, these tumors are not eliminated by the immune system, despite the expression of a number of different “non-self” viral gene products, indicative of effective tumor immune evasion. Frequently the malignant state is preceded by aberrant viral lytic replication, as indicated by increased antibody titers to lytic antigens prior to or at diagnosis.\textsuperscript{20} This implies that tumor progression stimulates antibody responses to lytic EBV components as a consequence of viral multiplication in the individuals, despite the tumor cells expressing mainly viral latency-associated gene products. Restricted EBV lytic expansion is possible because of the virus ability to overcome local immune surveillance due to various strategies of viral immune escape operating during the lytic cycle.\textsuperscript{21,22} Key viral functions play a critical role to impose survival and replication on genetically damaged cells, creating a basis for malignant development.\textsuperscript{23-25}

Although EBV is the strongest risk factor for NPC, as mentioned above, there are additional environmental factors which are also closely linked with the disease. Food such as salted fish, other preserved foods and herbal medicine have been linked to NPC. Several compounds in food have been observed to induce \textit{in vitro} EBV reactivation suggesting their capability of triggering enhanced \textit{in vivo} virus replication. Tobacco smoke, passive smoke, alcohol consumption, occupational dust and other inhalants are other non-viral factors related to NPC. Family history is also
among risk factors observed to be associated with NPC. First-degree relatives of NPC patients have 6- to 19-fold excess risk of developing the disease compared to those without any affected NPC case in the family.\textsuperscript{26,27} Aberrant EBV reactivation within family members was also observed among close family members of NPC cases,\textsuperscript{28,29} indicating that both genetic and environmental factors responsible for the aberrant EBV IgA are shared in the family.

Promoter DNA methylation of CpG islands is widely considered to be an important epigenetic mechanism in carcinogenesis and is one of the earliest and most frequent alterations leading to cancer:\textsuperscript{30,31} It is an important epigenetic mechanism for tumor suppressor gene (TSG) silencing, which may confer tumor cells of growth advantage.\textsuperscript{30,32} However, methylation is not randomly distributed in carcinogenesis and gene and cancer type specific.\textsuperscript{33-35} The specific patterns of CpG island hypermethylation may provide a useful signature for early tumor diagnosis and prognosis.\textsuperscript{35,36,37} In NPC gene silencing by promoter methylation has been shown for multiple tumor suppressor genes, each of which potentially contributes to the multistep oncogenesis. Many cellular pathways are inactivated by this epigenetic event including DNA repair, cell cycle, apoptosis, cell adherence and Wnt-signalling pathway.\textsuperscript{38} To define the value of promoter methylation of many TSGs for detection of early-stage NPC, a comparison among NPC patients, healthy EBV carriers and a high risk population is needed.

A successful tumor downstaging program needs to focus on a well-defined population at risk. Epidemiology studies pointed out three groups of population at high risk for developing NPC. The first group comprises people presenting with aberrant EBV IgA with a pattern similar to NPC.\textsuperscript{20,39-41} This population may develop NPC in months to several years entering the so-called serologic window. The second group consists of multiplex NPC family members.\textsuperscript{42,43} Screening by EBV IgA identified many cases of NPC either at enrollment or during follow-up. Within multiplex families, an elevated EBV IgA pattern was more frequently observed compared to the general population.\textsuperscript{28} The third group is formed by individuals with a history of chronic nasal and otological (or neurological) disease, who are proven to have increased risk of NPC compared to the cases without such history.\textsuperscript{44,45} The populations mentioned above may serve as high risk targets for setting up screening programs. Unfortunately, in Indonesia, there are no data on individuals showing elevated antibody to EBV with a similar pattern as NPC cases, because seroepidemiology has never been conducted. Targeting family members of multiplex NPC cases is not possible since only small number of multiplex NPC is found in our region. (Hariwiyanto et al., unpublished data) However, family members of scattered NPC may also be a significant target for case finding programs in population setting. This group of healthy subjects would also be well-motivated compared to individuals without any affected NPC case in their family. Regarding the insidious early symptoms of NPC which very rarely bring individuals to medical attention, people presenting with chronic symptoms in head and neck can be another target for screening in the clinical setting. Serological studies have not yet been done on
individuals with chronic symptoms in head and neck, therefore they will also be a focus of interest. Consequently, in this thesis first-degree relatives of NPC patients and people presenting with chronic complaints in head and neck areas are target groups for defining screening feasibility. A panel of tests for detecting aberrant EBV activity, as described below, was recently developed and optimized specifically for our population and is here evaluated in these groups.

Another key issue for a good screening program is the availability of non-invasive, low-cost screening tests that can detect disease at an early stage. Prior studies have indicated that EBV-related markers can be used for early detection (screening) and prognostic/monitoring. Our group has developed EBV serological tests, including IgA-based ELISA and IgG immunoblotting and EBV DNA viral load testing aiming for improved diagnostic tools in Indonesia. The molecular complexity underlying IgG and IgA anti EBV antibody responses in NPC was analyzed and a cheap, but sensitive and reliable one-step IgA ELISA format was established as NPC screening tool. This ELISA assay was then further modified to allow the usage in a simple finger prick dried blood sampling instead of venous blood collection.

Because NPC subjects showed stronger and more diverse IgG and IgA responses toward different types of EBV antigens, EBV immunoblot diversity provides a significant diagnostic value for discriminating between NPC and non-NPC tumors. When immunoblot was used as a confirmation test for the above mentioned one-step ELISA, the sensitivity and specificity for NPC diagnosis reached 98.0% and 99.2%, respectively. Furthermore, EBV DNA in nasopharyngeal brushings provides a non-invasive and useful method for showing the presence of EBV in situ at the nasopharyngeal site. Compared to histological analysis of invasive biopsy specimens, this test proved also convenient for long-term monitoring allowing repeated testing. Moreover, this sampling method may be conducted with or without nasendoscopy guidance. The EBV DNA load in nasopharyngeal brushing, as determined by quantitative real-time PCR, has good diagnostic value and was proposed as confirmation test for serology. However, since PCR requires clean lab space and equipment and also is considered expensive for screening in developing countries, this method was proposed as third option besides one-step ELISA and IgG immunoblotting.

This thesis describes the analysis of populations at risk for NPC using EBV-based assays. The core family members of NPC cases were tested using EBV IgA serology and the seropositivity was compared to age-, sex- and location-matched healthy controls of NPC cases without any family history of NPC. The utility of the ELISA-modification, using easier finger prick dried blood sampling, was tested in a population-based home-visit program when the individuals refused to undergo venous blood sampling. A comprehensive questionnaire capturing demography data and life-style habits of all participants was also performed to determine the exogenous factors which may influence the seroreactivity among the first-degree relatives. This also aimed to verify some life habits which may predispose to the
presence of antibody anti-EBV. As second population, a group of patients presenting with chronic symptoms in head and neck areas was tested for EBV presence similar as NPC patients. The 3-step approach was used in a scheme proposed previously. (Hutajulu et al., 2011, manuscript submitted) EBV IgA ELISA was used as the first screening test, considering its relative low cost. The result was confirmed by IgG immunoblot. EBV DNA load in nasopharyngeal brushings, which were collected at the initial intake, was tested only in the seropositive cases (cost reason), providing a third marker, independent of serology.  

Given the close association with carcinogenesis and its typical expression in epithelial cells, analysis of the BARF1 gene sequence and immunogenicity may provide information for development of improved diagnostic assays. Aberrant EBV serology is commonly used to support NPC diagnosis, and antibodies targeting the BARF1 protein could form a new diagnostic tool. RNA transcription of the EBV BARF1 gene was found to be selectively related to EBV-associated malignancies of epithelial origin. Recently plasma associated BARF1 protein was considered as a diagnostic marker based on observations of its active secretion of BARF1-expressing cell model systems and its detection in sera of NPC patients. This expression and secretion of BARF1 in human carcinoma suggest that BARF1 might trigger antibody responses, which have been poorly studied in the past.

Epigenetic-based tests, in particular gene promoter methylation, has been widely used for early cancer detection in several types of tumors, but is limitedly explored in NPC. The DNA methylation pattern may also serve as diagnostic marker for NPC. A selection of such epigenetic markers was evaluated for its complementary role in NPC diagnosis, with possible added value to EBV-based markers. Finally a modification, using both EBV DNA and methylation markers tested simultaneously in a methylation-specific PCR, was applied to assess its diagnostic value in parallel with obtained brush and biopsy materials from defined NPC cases.

**EBV-parameters tested in defined populations at risk for NPC**

Using the one-step EBV IgA ELISA, first-degree relatives of sporadic NPC cases showed significantly more frequent seroprevalence than healthy general population, in particular also having higher levels of seroreactivity (>3 times standard cutoff value/CoV) (chapter 2). This suggests that family members demonstrating elevations in higher seroreactivity of EBV IgA can be suitable candidates for further NPC screening. The EBV IgA seroreactivity above the high CoV is an appropriate discriminatory point for initiating clinical and serological follow-up and more intensive screening. This is the first study showing the presence of aberrant EBV serology in family members of sporadic NPC cases and adds to earlier observations in family members of multiplex NPC cases. It is possible that elevated seroreactivity observed among close family members of NPC reflects shared environmental exposure to EBV unrelated to disease risk. This is supported
by studies demonstrating that spouses of NPC cases also showed elevated antibody levels against EBV.\textsuperscript{28,29} Considering that spouses are genetically unrelated to the affected cases, shared environmental factors are likely important determinants of seroreactivity. However, a genetic component to the observed familial aggregation cannot be ruled out based on our data. Furthermore, this study implies that multiple EBV reactivation or re-infection may occur more frequently within NPC families compared to the general community. Therefore, genetic and environment can be considered as synergic risk factors for NPC, as suggested before.\textsuperscript{27}

A total of 217 individuals with chronic complaints presented in head and neck were recruited and tested using the 3-step approach of EBV-based laboratory tests (\textit{chapter 3}). At intake, as many as 75 individuals (34.6\%) demonstrated IgA seropositivity against EBV for the main marker, EBV IgA ELISA, of which 23 individuals (10.6\%) also showed strong antibody responses (above 3x CoV). Sixteen cases (7.4\%) or 21.3\% of the seropositive subgroup showed high IgG immunoblot score against EBV antigens. In these double seropositive individuals (16), 13 brushings were available for testing and high viral loads were revealed in eight cases. When submitted to clinical examination, seven cases revealed a nasopharyngeal mass on CT-scan. Three subjects were histologically confirmed as NPC.

Many seroepidemiology studies of NPC were performed in unselected healthy subjects or in high risk multiplex families. The present study is the first to determine serologic pattern against EBV in a group of individuals presenting with chronic symptoms suspicious of NPC. Three NPC cases (1.4\% of total individuals) were identified, two of them presented as early NPC cases. The three cases were firstly identified by EBV IgA ELISA and IgG immunoblot score confirmed the seropositivity. All of them showed elevated seroreactivity at enrollment not only above the standard CoV, but also above the cutoff of strong response which more closely resembles the NPC pattern. One available brushing from these cases proved to have high level viral DNA load. The one patient identified at relatively late stage (III) may illustrate the lack of awareness of our local community regarding symptoms associated with NPC. Thus, a specific education is needed and an active screening program using a community-based approach should be designed.\textsuperscript{60} Nevertheless, the goal of facilitating early treatment and better survival were achieved for these new cases by the case finding approach presented here. Otherwise in these individuals the growing tumor would have possibly remained unnoticed. Ji et al. (2007) also observed that even by routine screening 32\% of NPC patients were diagnosed in late stages although screening has decreased the frequency from 79\% prior to the program.\textsuperscript{29} Monitoring in follow-up has now been done every 6-12 months per case in 129 patients from our population and fluctuations of the serologic pattern has been detected during this follow-up, similar to prior studies.\textsuperscript{20,41} Our serial follow-up has not yet detected any new NPC case, but our study comprises a relatively short follow-up time compared to the prior ones.

From our studies in \textit{chapter 2 and 3} a strategy of using one (cheap) serology assay (EBV IgA ELISA) for initial screening, combined with additional EBV marker
panels shows promise for further screening attempts. Considering EBV IgA serology alone, seropositivity was demonstrated in 323 individuals (39.3%) out of the total 823 individuals studied in chapter 2 and 3. The 3-step assay was then applied to patients with chronic symptoms (chapter 3) revealing three new NPC cases, indicating its value for NPC early detection. Application of the 3-step approach for family members is limited by high cost and the need to undergo nasopharyngeal sampling. In both studies the use of dried blood filter paper sampling was proven feasible as suggested before. Its application can be easily implemented in the clinical setting in regional and remote hospitals as well. For individuals refusing to undergo venous blood sampling, this is a good alternative and promising later usage in a larger mass screening. When being (strongly) positive in a first serological analysis, subsequent brush sampling may be proposed for individuals refusing a first brush sampling.

To formally evaluate whether EBV serologic responses measured among healthy family members from sporadic NPC cases and individuals with chronic symptoms are predictive of subsequent NPC risk and to define whether EBV serology can be used clinically as a screening tool, all individuals with and without altered EBV serologic profiles need to be followed-up for incident NPC. However, such a mass screening is limited by cost and other lack of supports in our local situations, thus not feasible at this moment. Therefore, the focus on monitoring for case finding can be prioritized to include only those with elevated antibody, bearing in mind that seropositive subjects have higher risk for NPC development compared to seronegative persons.

An ideal screening tool should have both high sensitivity and specificity. EBV IgA ELISA alone have sensitivity and specificity of 90.1% and 85.4%. Confirmed with IgG immunoblot, EBV IgA ELISA showed increased diagnostic accuracy to 98% and 99%, respectively, in the Indonesian NPC samples. Sensitivity and specificity of using EBV DNA for NPC diagnosis were 98% and 90%, respectively. The diagnostic value of a combination of EBV IgA ELISA and EBV DNA load for diagnostic purpose has not been analyzed. When tested in a defined panel of NPC cases sensitivity of both serology and DNA load testing is similar (88.6% and 89.7%, respectively). (Thesis Fachiroh 2009) Considering the high sensitivity and specificity as well as rapid processing of nasopharyngeal brushings for EBV DNA load measurement, it was suggested that brush EBV DNA analysis could be used for primary diagnosis, as a complementary test for EBV serology to improve accuracy of screening NPC.

In this thesis EBV IgA ELISA was chosen as the reference test because of its high sensitivity for NPC diagnosis and the relative abundance of data relating to this marker. This assay is also relatively cheap compared to other markers (chapter 3) especially when applied in an extended case finding program. Thus, the EBV IgA has been tested in three populations in our present studies, i.e. (group 1) first-degree relatives of NPC versus (group 2) healthy controls of NPC cases (chapter 2) and (group 3) individuals presenting with chronic complaints in head and neck (chapter 3). Taken together, the seroprevalence of elevated antibody
was about similar across the three groups, 41.2%, 39.5% and 34.6%, respectively. To discriminate low-level seroreactivity, a cutoff point distinguishing strong responders from weak responders was determined across the population of family members versus general healthy controls and set at $OD_{450} = 1.000$ (approximately 3 times of the COV). This point gave significant difference between both groups (chapter 2). Using this higher COV, the seropositivity of EBV IgA among the three groups was 15.2% (79/520), 5.6% (5/86) and 10.6% (25/217), respectively. This provided significant difference between the proportions of all groups ($p=0.041$). Therefore it is inferred that both family members of NPC and individuals presenting with chronic symptoms are at the similar level of risk for EBV serodetection, and possibly further for NPC development.

Both IgG immunoblot and EBV DNA load confirmed the aberrant EBV result of EBV IgA ELISA before clinical confirmation of NPC (chapter 3). All three NPC cases identified showed high scores for IgG immunoblot and the available brushing showed a high level of EBV DNA. The small number of seropositive subjects did not allow these parameters to be statistically verified as a confirmation test for case finding of NPC. However, our overall results indicate that the 3-step EBV-based testing, using in-house standardized and relatively simple and cheap tests, can serve as a promising approach for further extensive NPC risk screening and follow-up monitoring.

### EBV-BARF1 sequence diversity and use of antibody anti-BARF1 for NPC diagnosis

The viral BARF1 protein is considered as an important EBV oncogene and RNA encoding BARF1 was detected in most NPC isolates. Unlike the viral oncogene LMP1, in which multiple sequence and functional variations have been described, the information on the BARF1 sequence variation is limited. Chapter 4 describes BARF1 sequence variations among EBV isolates derived from NPC cases, healthy EBV-carriers and other EBV associated disorders. Immunogenic properties of NPC derived BARF1 as a purified hexameric protein was also determined (chapter 4 and 5).

Most NPC isolates demonstrated specific BARF1 nucleotide changes compared to prototype B95-8 virus (chapter 4). A significant proportion of NPC isolates showed at least one amino acid conversion (80.3%) compared to non-NPC isolates (33.3%) ($p<0.001$). However the sequence variation was not significant when NPC was compared to BARF1 sequences in LCLs established from direct blood Bcell cultures of Indonesian healthy donors (40.0%) ($p=0.074$). The BARF1 genetic diversity to EBV genotype, viral load and serology markers was not detected. This suggest that the BARF1 sequence found in most (Indonesian) NPC samples reflects the prevalent regional EBV strain, rather than a specific NPC oncogenic subtype. Using the published crystal structure of hexameric B95-8 derived BARF1, possible
implications of the most prevalent amino acid conversions on protein secondary structure elements was analyzed. The conserved V29A mutation is positioned at the start of an internal beta-sheet element and is predicted to render only small changes, if any, to the overall structure. The W29G change is providing more flexibility to a beta sheet element inside the BARF1 molecule normally stabilized by hydrophobic interactions. It is uncertain to what extent this substitution affects the overall BARF1 structure. The conserved H130R mutation is located towards the water-contact surface of the inner core of the BARF1 hexameric structure and may have little effect on structure, merely affecting hydrophilic water interactions. Overall, the most prevalent amino acid changes in BARF1 sequence are therefore not considered to affect the three-dimensional structural properties of hexameric BARF1 protein significantly (chapter 4).

Furthermore, chapter 5 described the expression and purification of NPC-derived BARF1 with sequences of two conserved and most prevalent mutations, V29A and H130R. This mutant BARF1 protein was expressed and secreted as a hexameric molecule in human HEK293 cells and could be purified to homogeneity by lectin-affinity chromatography. The purified native hexameric BARF1 protein was used to determine whether the BARF1 mutation might have a direct influence on immune recognition and possible immune escape. However, the humoral immune responses against prototype BARF1 (B95-8) and purified native hexameric BARF1 in sera of Indonesian NPC patients, healthy EBV-positive and EBV-negative individuals were relatively weak. The IgG and IgA responses in human sera, which were much weaker than antibody responses to the immunodominant antigens of EBV (EBNA1 and VCA-p18). However, a significant higher IgG reactivity was found in NPC patients as compared with Indonesian EBV-positive and EBV-negative healthy controls. From these results it is indicated that BARF1 is a relatively weak immunogenic protein in humans, even when having the NPC-specific genetic variation. The use of the native hexameric BARF1 protein proved to be important in detecting anti-BARF1 antibody responses in NPC patients. Thus, antibody response against BARF1 antigen has limited value for NPC diagnosis. However, the detection of BARF1 as a secreted tumor marker protein in serum/plasma is an option for further study, as suggested by Houali et al. (2007).56 New monoclonal antibodies for BARF1 capture were recently developed and proteomic fingerprints were determined (Hoebe, Hopmans and Middeldorp, unpublished data), but preliminary data do not confirm the high levels of circulating BARF1 as suggested by Houali et al.. More effort is needed to define whether secreted BARF1 can be considered a potential circulating NPC tumor marker.
DNA methylation as complementary marker aside EBV-based parameters for NPC diagnosis

Using a simple methylation-specific PCR, the (parallel) analysis of multiple gene methylation possibly involved in NPC development is proposed as a complementary test for NPC risk assessment (chapter 6). Methylation analysis on brushing samples of NPC cases compared to a high risk group and EBV-positive healthy carriers has identified TSG to be associated with NPC cases. TSG promoter methylation analysis demonstrated good diagnostic value when using a panel of five methylated genes that were frequently methylated in NPC samples. This panel consists of RASSF1A, p16, WIF1, CHFR and RIZ1 genes playing role in the regulation of apoptosis, cell cycle and mitotic-checkpoint, cytoskeleton organization and Wnt-signalling pathway. Several other candidate markers identified in prior Chinese studies proved also frequently methylated in non-NPC groups in the Indonesian population. Methylation of at least one of the five TSGs provides sensitivity and specificity of 98% and 96% in discriminating NPC case from the non-cancer subjects. By using only two markers (RASSF1A and p16 gene) the sensitivity and specificity already reached 91% and 96%. When compared with serology tests using IgA/[EBNA1+VCA-p18] and viral DNA load, there was no correlation found between these EBV-based parameters and aberrant methylation of the five TSGs. This indicated that methylation is an independent marker that may provide added value to the EBV-based markers in NPC screening. For a developing country like Indonesia a methylation-specific polymerase chain reaction (MSP) assay is a relatively economical and can be suitable for screening in a large population. The less-invasive procedure of brushing sampling rendered comparable (81.8%) methylation detection rate when compared to the corresponding paraffin biopsy tissue analysis, underlying its role as good sampling when applied in an early detection program.

A newly defined TSG, MAL, considered to play a role in viral-epithelial malignancy yielded promising indications for its use as methylation marker for early NPC detection. This study is the first to explore promoter methylation of the MAL gene in NPC. The detection of methylated MAL is quantitative using a CoV to discriminate NPC from healthy individuals, which enables a highly specific and sensitive assay. For using the assay in a qualitative assay which is more cost effective further optimization is needed.

Chapter 7 describes an assay that provides multiplex information in a single PCR reaction with small DNA samples. It is an elegant combination of PCR detecting methylation of NPC-related risk genes and EBV. The panel of markers consist of two cellular TSGs including RASSF1A and DAPK1 and two unmethylated markers of EBV, EBNA1 and LMP1. This method has proven to be informative and require small clinical materials. Swab isolates revealed a 98% and 100% NPC detection
rate compared to the corresponding biopsy. This provides a promising methylation marker approach for population screening and early diagnosis for NPC. Its feasibility for early-stage NPC detection requires further exploration in a defined-population at risk.

Importantly, although DAPK1 methylated gene worked very well in this multiplex MSP (MMSP) assay, DAPK1 methylation proved an unreliable marker for NPC in Indonesian cases (chapter 6) causing this gene to be excluded from the panel of multiple methylated genes using simple MSP. Different primers designed from different methylated regions of DAPK1 in both tests might have caused the different outcomes, but was not further explored due to time constraints.

**Environmental factors may relate to seroreactivity and epigenetic alteration**

Environmental exposures are observed to influence seroreactivity against EBV antigen\(^\text{28}\) and epigenetic events.\(^\text{68,69}\) The study on family members of NPC showed that some risk factors had a quite strong association with EBV IgA seropositivity among NPC family members. Current tobacco chewing, consumption of grilled food at least once a week, consumption of salted fish at least once in a week, exposure to smoke in the household, consumption of instant noodle and exposure to passive smoking sparingly in a year were associated with higher odds of seropositivity. The ORs were 2.61, 1.77, 1.75, 1.57, 1.53, 1.31, respectively, although the 95% CI did not support the significance (chapter 2). In this population-based study when family members were compared to the general community it suggests a role of familial and environmental factors’ in the reactivation and reinfection of EBV. The exogenous factors mentioned above have never been reported as risk factors for NPC development in our local population. A small scale case-control study has been done in our local population but the small sample size did not allow a good statistical analysis. A bigger and more extensive case-control study is at present being set-up to explore the possible risk factors for NPC. (Fachiroy et al, study in preparation)

Persistent exposure to adverse environmental carcinogens and general toxins can alter gene expression patterns in a heritable manner and act as an initial step preceding malignant transformation.\(^\text{70,71}\) EBV infection, which has potential effect on DNA methylation, chromatin organization and histones\(^\text{71}\) may enforce these effect and initiate potential NPC growth. Indeed reports in the local population indicate that diet might predispose towards cancer development. Formaldehyde, boric acid, Rhodamine B, and yellow Metanyl are among many observed chemicals compounds found in the food of local markets in Indonesia. (Kompas 2006, Sydney Morning Herald 2006) Our data showing high frequency of methylation in some TSGs in the Indonesian healthy population may be related to such daily environmental exposure. However, this should be interpreted carefully since Dutch healthy EBV carriers also showed a similar pattern of methylation. The potential relationship between carcinogenic exposure and promoter methylation status is subject to further study.
Future perspective

An extensive clinical and serological follow up involving EBV IgA seropositive individuals known to have higher risk for NPC (family members, people with symptoms) should be designed. EBV-parameters tested in both populations showed that EBV IgA ELISA is indeed an economically affordable and clinically useful first screening marker, either performed on serum sampling from venous blood or the finger prick dried-blood modification. EBV DNA detection in nasopharyngeal brushing and methylation analysis of multiple NPC-related TSGs were demonstrated as promising complementary markers to this initial EBV-based serological screening assay. A multiplex test including methylation analysis together with EBV detection is currently under investigation. For serological confirmation the IgG immunoblot diversity test \(^{48,72}\) or the more recently described and more simple EAIgG/IgA ELISA \(^ {73}\) may be used in the near future. The quantity of viral DNA in blood or plasma remains controversial, because of generally low levels of circulating EBV-DNA in NPC patients. \(^ {74}\) Antibody response to BARF1 is not a very useful marker, because of the low immunogenicity of BARF1, but the detection of BARF1 as a secreted protein in the circulation is an option for further study. The use of multiple independent markers with complementary value may be the best option for early stage NPC screening, but financial constraints have to be considered in developing countries. Screening study participants with defined but unexplained suspicious symptoms and/or a family history of NPC will have a high compliance to the screening program, because of inherent self-motivation. Increasing awareness on NPC and its common signs and symptoms among the head and neck medical specialists, \(^ {60}\) and spreading information on - and increase use/availability of- relevant sampling and novel diagnostic testing opportunities may improve early stage identification of NPC in the general population in Indonesia.

Other work that is needed for increasing patient survival are to improve post-treatment monitoring for recurrent disease and distant metastasis and avoid loss to follow-up, by improving medical registries and documentation (e.g. via an online data system) \(^ {75}\) and improving medical counseling. Further research, including basic risk factor analysis, should seek to specify risk factors present in the local Indonesian population and to identify particular compounds in preserved foods that contribute to the pathogenesis of NPC. Integrated health community education should aim to achieve better awareness in public community for early symptoms of NPC (and cancer in general) first targeting the NPC family members and individuals with suspicious symptoms in the general community. The most feasible means of lowering NPC risk in the population would be avoiding carcinogen exposure that may endanger the population to cancer in general. All above measures should contribute to prevention and downstaging of NPC tumors at presentation.

A key to a successful patient care program is to improve treatment facilities, especially radiotherapy, and combining effective treatments resulting in long term survival. Current chemoradiation protocols should be optimized and overall
compliance with therapy schedules is a first priority. Photodynamic therapy is at present used to manage local residual disease (Indrasari and Tan, study in progress) and EBV-lytic induction is later introduced to advanced metastastatic patients. (Tan et al., 2011, study in preparation) Progression in case of residual or recurrent disease can also be detected in early phase by introducing EBV-parameters as prognostic factors during and following NPC treatment. (Adham et al., 2011, submitted) (Taroeno-Hariadi et al., 2011, study in progress)

This study has built on prior developed EBV-based methods for NPC diagnosis and showed their feasibility for more wide-spread use in Indonesia. (Thesis Fachiroh 2009 and Paramita 2010) The addition of methylation marker analysis is advocated by this study. New markers will certainly (need to) be discovered reflecting early events in the EBV-driven and environmentally enhanced carcinogenic accidents leading to NPC. Only prevention and early-stage recognition of NPC will be economically feasible options in the near future, in a country that cannot afford to build multiple specialized chemo-radiation centers for its wide population. Search for (therapeutic) vaccination and preferably oral (virus-) specific NPC treatment at early stage is a final option to reduce the burden of NPC in highly endemic regions of the world, including Indonesia.

References


